SAPK/JNK Activation and Apoptotic Induction by the Macrophage P2X7 Nucleotide Receptor

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Abbreviations: KN-62, 1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; Bz, 3’-O-(4-benzoyl)benzoyl; LPS, lipopolysaccharide; IL-1β, interleukin-1β; TNFα, tumor necrosis factor-α; 2-MeS-ATP, 2’-methylthio-ATP; oATP, oxidized ATP; MTX, maitotoxin, MTB; *Mycobacterium tuberculosis*. 
Summary

In human and rodent macrophages, activation of the P2X7 nucleotide receptor stimulates interleukin-1β processing and release, apoptosis, and killing of intracellular Mycobacterium tuberculosis (MTB). Signaling pathways downstream of this ionotropic ATP receptor are poorly understood. Here we describe the rapid activation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway in BAC1 murine macrophages stimulated by extracellular ATP. Brief exposure of the cells to ATP (10-30 minutes) was sufficient to trigger a rapid accumulation of activated SAPK that was then sustained for over 120 minutes. Several observations indicated that the P2X7 receptor mediated this effect. 1) ATP and BzATP were the only agonistic nucleotides. 2) The effect was inhibited by oxidized ATP and the isoquinoline KN-62, two known P2X7 receptor antagonists. 3) ATP-induced SAPK activation could be recapitulated in P2X7 receptor-transfected HEK293 cells but not in wild type HEK293 cells. Because P2X7 receptor stimulation can rapidly activate caspase-family proteases that have been implicated in the induction of the SAPK pathway, we investigated whether ATP-dependent SAPK activation involved such proteases. Brief exposure of Bac1 macrophages to extracellular ATP induced DNA fragmentation, alpha-fodrin breakdown, and elevated levels of caspase-3 type activity. DEVD-cho, a caspase-3 inhibitor, inhibited ATP-induced DNA fragmentation and alpha-fodrin proteolysis, but had no effect on ATP-induced SAPK activation. YVAD-cmk, a caspase-1 inhibitor, prevented ATP induced release of processed IL-1β but not ATP-dependent SAPK activity. We conclude that activation of ionotropic P2X7 nucleotide receptors triggers a strong
activation of SAPK via a pathway independent of caspase-1 or caspase-3-like proteases.

**Introduction**

The P2X7 nucleotide receptor belongs to the P2X family of ATP-gated ion channels. This family comprises seven distinct gene products, each possessing two putative membrane-spanning domains with intracellular N- and C-termini (for review see 1, 2). The P2X7 receptor is identical to the functionally defined P2Z receptor and is expressed primarily in hematopoietic cells and a limited number of other cell types including parotid acinar cells, testis and fibroblasts (3-5). As a non-desensitizing, nonselective cation channel with low affinity for ATP, P2X7 receptor activation requires millimolar extracellular ATP in the presence of divalent cations. Channel opening triggers rapid depolarization, calcium influx and equilibration of sodium and potassium gradients. Because individual P2X7 receptor proteins contain only two transmembrane spanning segments, it is assumed that the functional channels are oligomeric complexes composed of several individual subunits. Recent studies indicate that recombinant P2X7 receptor subunits can self-assemble during *in vitro* translation and processing into stable, detergent-resistant complexes (6). P2X7 receptor activation additionally induces a non-selective pore able to pass molecules up to 800 Da, a characteristic shared to a lesser degree by other P2X members (7, 8). Pore structure remains uncharacterized. The ability of heterologously expressed P2X7 receptors to reconstitute ATP-dependent channel/pore formation has been interpreted as evidence that the pore reflects either further multimerization of the P2X7 channels or a dynamic change in the selectivity filter of
P2X7 channels (9). Other data suggests that the channel and the pore are separate entities (10).

Depending on cell background, activation of the P2X7 receptor triggers diverse physiologic processes. For example, human monocytes primed by bacterial endotoxin/lipopolysaccharide (LPS) respond to extracellular ATP with the caspase-1-dependent proteolytic maturation and externalization of IL-1β (11-13). Apoptosis is another prominent consequence of P2X7 receptor activation in various types of leukocytes. Zanovello et al. first demonstrated ATP-dependent apoptosis in a lymphocyte cell line (14) and Zheng et al. confirmed this observation in murine thymocytes (15). Hogquist and coworkers later showed that brief (30 min) exposure of thioglycollate-elicited murine peritoneal macrophages to ATP was sufficient to initiate the signaling cascade that leads to apoptotic death (11). Subsequent studies have specifically implicated the P2X7 receptor in mediating ATP-induced apoptosis of human macrophages, mesangial cells, dendritic cells and microglial cells (16-19). As with most examples of apoptosis, the P2X7 receptor-initiated cascade involves a defined sequence of phenotypic changes that culminate in death only several hours after the transient exposure to ATP. Agonist-occupied P2X7 receptors also drive signals that induce nuclear accumulation of various activated transcription factors, such as NFAT within minutes or NFκB within hours (20, 21). The P2X7 receptor-induced proteolytic processing and release of IL-1β from LPS-primed macrophages also precedes cell death (13). These observations suggest that in the time period between commitment to apoptosis and actual cell death, P2X7 receptor activation triggers additional signals, such as NFAT activation or IL-1β release, that
modulate the overall inflammatory response of macrophages. This possibility is supported by the observation that brief ATP pulses trigger not only macrophage apoptosis but also killing of intracellular mycobacteria, including virulent *Mycobacteria tuberculosis* (MTB) (22). Because other inducers of macrophage apoptosis do not kill MTB, an as yet unidentified P2X7-specific signal presumably induces killing of internalized MTB before the macrophage itself dies (16, 23). The biochemical steps linking channel/pore activation to IL-1β release, accumulation of pro-inflammatory transcription factors, MTB killing, and macrophage apoptosis are poorly understood.

The stress-activated protein kinases [SAPKs, also known as cJUN NH2-terminal kinases (JNKs)] phosphorylate and activate transcription factors such as ATF2, Ets and cJUN in response to diverse cell stressors. These include UV radiation, osmotic shock, inflammatory cytokines and endoplasmic reticulum stress (for review see 24, 25). Many of the downstream effectors of SAPK signaling contribute to the inflammatory response, including the TNFα-dependent induction of E-selectin, NFkB induction in T cells, and both pro-apoptotic and anti-apoptotic effects in a variety of cell types (24). A necessary role of SAPK in apoptotic induction by UV irradiation, but not Fas receptor ligation, was demonstrated in a recent study using embryonic fibroblasts derived from double-knockout mice that lack expression of both the JNK1 and JNK2 genes (55).

Proximally, SAPKs are activated by the dual-specificity MAP kinase kinases (MEKs) which in turn are activated by the MEK kinases (MEKKs). Upstream regulators of MEKK are incompletely characterized. TNFα-dependent SAPK activation is best described and involves recruitment of the adaptor protein TRAF2 to the cytosolic portion
of the ligated TNFα receptor. TRAF2 mediates the activation of a series of downstream kinases that leads to phosphorylation of SAPK itself (26). Other intermediates have been proposed to play a role in different models of SAPK activation including oxidative stress, DNA damage and caspase proteases. Altered ion fluxes have also been associated with SAPK activation in several systems. Kuroki et al. described activation of SAPK by palytoxin, a natural marine toxin from Palythoa tuberculosa that acts as a skin tumor promoter and modulator of the Na⁺,K⁺-ATPase (29). Moreover, palytoxin-induced SAPK activity requires sodium flux (30). Similarly, UV irradiation of myeloblastic leukemia cells induces a prominent K⁺ channel activation that, in turn, induces SAPK-dependent apoptosis (31). Since a major consequence of P2X7 receptor activation is bulk movement of both sodium and potassium, we hypothesized that P2X7 receptor activation would activate SAPK. We found that exposure of murine macrophages to short pulses of extracellular ATP can rapidly induce a sustained activation of SAPK. Pharmacological selectivity and molecular evidence indicated that the P2X7 receptor mediates this ATP-induced kinase activity. The P2X7 receptor can activate caspases involved in either cytokine processing or apoptotic induction (18, 19). Thus, we also evaluated the possible involvement of caspase-1 and caspase-3 in this P2X7 receptor-induced pathway of SAPK activation. The results indicate that although both caspase-1 and caspase-3 were activated by the pulsed ATP protocol, neither protease plays a role in coupling the P2X7 receptor to the SAPK signaling cascade.
Experimental Procedures

Materials – All nucleotides were from Sigma except for 2-meS-ATP which was from Research Biochemicals International (Natick, MA). Anisomycin and ouabain was from Sigma. The GST-Jun (1-79) plasmid was from Dr. J. Woodgett, Woods Hole Biological Laboratory. $[^\gamma-\text{32P}]$ATP was from NEN Life Science Products. Anti-SAPK and anti-phospho-specific SAPK antibodies were from Santa Cruz (Santa Cruz, CA). The anti-alpha-fodrin antibody was from Chemicon. KN-62 and the caspase-1 fluorogenic substrate peptide were from Biomol (Plymouth Meeting, PA). The capture and detecting antibodies used for the murine IL-1$\beta$ ELISA were from Endogen (Woburn, MA). DEVD-cho and YVAD-cmk were from Bachem. The caspase-3 fluorogenic substrate peptide was from Calbiochem. Recombinant murine TNF$\alpha$ and a neutralizing antibody against murine TNF$\alpha$ were from R&D Systems, Inc.

Cell Culture The BAC1.2F5 macrophage cell line, a clone of the SV40 transformed murine macrophage cell line BAC1, was maintained using previously described protocols (32). Wild type HEK293 cells and HEK cells stably transfected with the human P2X7 receptor were maintained as described previously (33).

SAPK Assay – JNK activation was measured according to Kuroki et al. (29, 30) with minor modifications. BAC1 cells ($3 \times 10^6$ /ml) were plated in 6-well dishes. Cultures were incubated with test agents dissolved in Iscove’s DMEM supplemented with 0.1% BSA at 37 °C. Samples were then washed once with cold PBS and lysed with lysis buffer (25 mM HEPES, pH 7.7, 300 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, pH 8.0, 0.1%
Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 2 µg/ml leupeptin, 100 µg/ml PMSF). Whole cell lysates were rotated for 30 minutes at 4 °C followed by centrifugation at 10,000 x G for 10 minutes. Supernatant protein concentration was determined by the Bradford assay. 50 µg of lysate was diluted to contain 20 mM HEPES (pH 7.7), 75 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 2 µg/ml leupeptin and 100 µg/ml PMSF and mixed with 10 µl of GSH-agarose beads (Sigma) bound to the GST-Jun fusion protein. The mixture was rotated for 2 hr at 4 °C and the beads were then washed three times in HEPES binding buffer (20 mM HEPES (pH 7.7), 50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Triton X-100, 0.1 mM Na₃VO₄, 2 µg/ml leupeptin and 100 µg/ml PMSF). Beads were resuspended in 40 µl kinase buffer (20 mM HEPES (pH 7.6), 20 mM MgCl₂, 20 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM Na₃VO₄, 2 mM DTT, 20 µM ATP and 5 µCi [γ-³²P]ATP) and incubated for 15 min. at 25° C. Proteins were eluted in SDS buffer, boiled for 3 min and separated by SDS-PAGE (12%). After Coomassie blue staining, the gel was dried and exposed to Kodak x-ray film (Eastman-Kodak, Rochester, NY). GST-Jun phosphorylation was quantitated with a Bio-Rad PhosphorImager.

Western Blots — Supernatant lysates were separated by SDS-PAGE (12%) and electrophoretically transferred to polyvinylidine fluoride (PVDF) membranes for 15 hours at 30 mV. PVDF membranes were rinsed in immunoblot buffer (10 mmol/L Tris,
pH 7.4; 0.9% NaCl; 0.05% Tween-20; 1 mmol/L EDTA) and blocked with milk buffer (4% nonfat dried milk (Sigma Chemical Co, St Louis, MO) in immunoblot buffer). After washing (1 x 15 min, 2 x 5 min) with immunoblot buffer, the PVDF membranes were incubated for 1 hour at room temperature with primary antibodies dissolved in immunoblot milk buffer. Both anti-JNK antiserum and anti-phospho-specific JNK monoclonal antibody was used at 1 µg/ml. Membranes were then washed and incubated for one hour with 1:5,000 dilutions of horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (for anti-JNK antiserum) or anti-mouse (for anti-phospho-specific JNK) antibody (Amersham, Arlington Hts, IL). Membranes were washed and developed using chemiluminescent reagents (SuperSignal from Pierce, Rockford, IL) for 0.5 to 5 minutes and exposed to Kodak x-ray film (Eastman-Kodak, Rochester, NY).

**Measurement of DNA fragmentation** – At appropriate time points, 1 x 10^6 cells (both floating and adherent) were centrifuged at 1500g for 30 seconds and resuspended in 0.5 ml lysis buffer (5 mM TrisHCL, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) and placed on ice for 15 minutes. Samples were then centrifuged at 12,000g for 20 minutes and the supernatant containing DNA cleavage products was precipitated overnight using isopropanol. Pellets were resuspended in TE and digested with proteinase K, 0.2 mg/ml and RNAse A, 1 mg/ml, for 30 minutes at 37°. DNA fragments were separated on a 1.5% agarose gel, visualized with ethidium bromide and photographed.

**Caspase-3-like activity** – After challenge with appropriate agonists, cell monolayers were washed once with 150 mM NaCl, 20 mM TrisHCL, 1 mM EDTA, pH 7.5. Cells were centrifuged for 30 seconds at 1500g and pellets were resuspended in lysis buffer (20
mM Tris HCL, 150 mM NaCl, 1 mM DTT, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, pH 7.5), incubated for 15 minutes at 37° and centrifuged at 12,000g for 20 minutes. The supernatants were then stored at -80° for future use. To assess supernatant caspase-3-like activity, 0.3 ml lysate was combined with buffer (100 mM NaHEPES, 10% glycerol, 1mM EDTA, 5 mM DTT, pH 7.5) and fluorogenic caspase-3 substrate to a final concentration of 14 μM. Fluorescence was measured at an excitation wavelength of 380 nm and an excitation wavelength of 460 nm at 10-minute intervals using a fluorescence spectrophotometer (Perkin-Elmer). Data was plotted and slopes calculated along the linear portion of the curve (at least five separate measurements). Data is presented as slope, in arbitrary units.

IL-1β ELISA - Macrophages were plated in 12 well dishes at 5 x 10^5/ml one day prior to challenge. On the day of the experiment, cells were stimulated with LPS (1 μg/ml) for four hours to induce expression of proIL-1β. In certain groups, the YVAD-cmk inhibitor was added for the final 30 minutes of LPS incubation. In the continued presence of serum-containing DMEM and LPS, cells were challenged with 3 mM ATP with or without the YVAD-cmk inhibitor. After 60 minutes, 5 μl aliquots of the tissue culture supernatant were assayed for IL-1β release by a sandwich ELISA. Briefly, a 96 well plate was coated with 1 μg/ml primary anti-murine IL-1β overnight, then blocked with 4% BSA in PBS for 1 hour. Plates were washed 3 times with wash buffer (50 mM TrisHCL pH 7.5, 0.2% Tween-20). 5 μl aliquots of tissue culture media or murine IL-1β standards, diluted with 45 μl of DMEM, were added to the blocked wells together with 50 μl of a second, biotinylated anti-murine IL-1β antibody (at 0.2 μg/ml). The plates
were incubated at room temperature for 2 hours and then washed 3 times. The captured immune complexes were colorimetrically detected by subsequent incubations with streptavidin-horseradish peroxidase (HRP) conjugate and tetramethyl benzidine (TMB) substrate for HRP.
Results

SAPK is activated by stimulation with extracellular ATP - One of the early effects of P2X7 receptor activation is equilibration of the trans-plasma membrane K\(^+\) and Na\(^+\) gradients. Since ionic perturbation can trigger SAPK signaling in certain cell types, we investigated the ability of P2X7 receptors to couple to SAPK. As an experimental approach, BAC1 murine macrophages were pulse-stimulated with extracellular ATP for relatively brief periods ranging from 10 to 30 minutes. Brief ATP pulses may be a more “physiological” stimulus since tissue macrophages are likely to be exposed to extracellular ATP for only short times due to the widespread expression of ecto-apyrases which enzymatically degrade extracellular ATP. Although removal of extracellular divalent cations can increase P2X7 receptor affinity for ATP, tissue macrophages are continuously exposed to millimolar levels of extracellular Mg\(^{2+}\) and Ca\(^{2+}\). For this reason, tissue culture medium (Iscoves’s DMEM) containing normal levels of divalent cations was employed in all experiments. In the experiment illustrated in Figure 1, BAC1 cells were pulsed with 5 mM ATP. After a 30-minute incubation the ATP-containing media was aspirated and replaced with normal ATP-free Iscove’s medium. At the indicated time points (Figure 1 A, B) cell lysates were assessed for SAPK activity. SAPK activity was low during or immediately following the ATP pulse but increased rapidly after ATP removal to a maximum of 4.6 fold over basal. The kinase remained activated even two hours after ATP washout. Since continuous ATP exposure can trigger colloido-osmotic lysis in these cells, control experiments verified that the 30-minute
ATP pulse itself did not cause necrotic cell death. The cytosolic enzyme lactate dehydrogenase (LDH) was used as a marker for lysis. No increase in LDH was observed in the extracellular medium at any point during ATP pulses (data not shown). Cells were harvested in subsequent experiments at 60 minutes following the ATP pulse since the induced SAPK activity was maximal at this time. To further characterize the SAPK response, BAC1 cultures were challenged with ATP for different times (0-30 min) prior to washout. Figure 2 shows that the magnitude of SAPK activation (measured at 60 min post-ATP removal) was dependent on the duration of the ATP pulse. A 5 min pulse was sufficient to elicit a measurable accumulation of active SAPK and near-maximal activation was observed within 20 min.

The P2X7 receptor mediates SAPK activation - BAC1 macrophages express calcium mobilizing P2Y receptors in addition to P2Z/P2X7 receptors (34) and given the high concentration of ATP used to activate SAPK we next determined whether the ATP-induced SAPK activity was mediated by the P2X7 receptor or by another P2 receptor. Figure 3A shows that preincubation of macrophages with periodate-oxidized ATP blocked ATP-induced SAPK activity. In contrast, there was no inhibition of anisomycin-induced SAPK activity, indicating that the SAPK signaling pathway remained intact after oATP pretreatment. Western blot analysis using an anti-SAPK antibody verified that the assay conditions did not alter
overall SAPK protein levels. Tyr and Thr phosphorylation of SAPK is required for activity and we verified that enzymatic kinase activity increased in parallel with accumulation of phospho-SAPK immunoreactivity (Fig. 3A). Although oATP inhibits the P2X7 receptor, its specificity for the P2X7R over other P2X receptors is unclear. Therefore, we verified that the isoquinoline derivative KN-62, a compound that also inhibits the P2X7 receptor in BAC1 macrophages (33), completely blocked ATP-induced SAPK activity (Fig. 3B). These pharmacologic data implicate the P2X7 receptor as the P2 receptor responsible for SAPK activation.

The ability of nucleotides other than ATP to activate SAPK was also tested. Figure 3C shows that only BzATP and ATP were SAPK agonists. Significantly, the P2Y2 agonist UTP was without effect, ruling out a contribution of these metabotropic P2 receptors expressed in BAC1 macrophages. The millimolar concentrations of ATP used to stimulate the P2X7 receptor causes strong chelation of extracellular divalent cations and this could contribute to the observed activation of SAPK. Since UTP also chelates divalent cations but does not activate P2X7 receptors, the failure of 5 mM UTP to simulate any SAPK activity suggests that simple chelation of extracellular divalent cations cannot explain ATP-dependent SAPK activity (Fig. 3C). The P2Y1 agonists 2-meS-ATP and ADP and the P1 agonist adenosine were also ineffective at inducing SAPK activity. This nucleotide selectivity profile is consistent with reported nucleotide with pharmacology of both cloned and natively expressed P2X7 receptors.
Macrophages can release various inflammatory cytokines, such as IL-1β and TNFα, which also activate SAPK signaling cascades, and these cytokines can act as autocrine modulators of LPS-initiated macrophage activation (56). The delayed, but sustained, activation of SAPK that followed ATP pulsing suggested an indirect mechanism that could involve the release and gradual extracellular accumulation of TNFα and/or IL-1β. However, the IL-1β and TNFα genes are transcriptionally silent in quiescent macrophages in the absence of primary inflammatory stimuli, such as LPS or interferon-γ (56). It should be stressed that P2X7 receptor-dependent activation of SAPK was observed in BAC1 macrophages even in the absence of priming by LPS or interferon-γ (Figures 1-3). Western blot analyses confirmed the absence of IL-1β or TNFα protein expression in either control BAC 1 macrophages or cells stimulated with ATP pulses in the absence of LPS pretreatment (data not shown); this contrasted with the marked accumulation of these cytokines in LPS-treated BAC1 cells. The delayed accumulation of phosphorylated SAPK triggered by ATP was not attenuated when BAC1 macrophages were incubated with neutralizing anti-TNFα antibodies, during and following ATP pulse stimulation (data not shown). Finally, as demonstrated in subsequent experiments (Figure 6 and Table 1), treatment of LPS-primed BAC1 cell with an inhibitor of interleukin-converting enzyme (ICE) / caspase-1 failed to attenuate ATP-induced SAPK activation despite strong inhibition of the ATP-induced IL-1β secretion. These studies indicate that release of inflammatory cytokines is an unlikely mechanism for induction of the SAPK signaling cascade by P2X7 receptors.

*Heterologous expression of the P2X7 receptor confers ATP-sensitive SAPK*
activity - To further investigate the relationship between P2X7 receptor activation and the stimulation of SAPK, we compared the abilities of ATP to activate SAPK in wildtype HEK293 and an HEK line that stably expresses the human P2X7 receptor (HEK-P2X7). To prevent detachment of cells during ATP stimulation, HEK cells were plated in poly L-lysine coated tissue culture dishes. Figure 4A demonstrates that P2X7 receptor-transfected cells, but not the untransfected (HEK-wt) cells, were characterized by a sustained elevation of SAPK activity following a 20-minute pulse with 5mM ATP. As a positive control, anisomycin stimulated SAPK equally well in both cell types. The accumulation of phosphorylated SAPK in HEK-P2X7 cells following the 20-minute ATP pulse was characterized by a timecourse (Figure 4B) that was similar to the timecourse observed in the BAC1 macrophages (Figure 1B), with maximal accumulation occurring ~60 minutes following the removal of ATP. These experiments provide definitive evidence that the P2X7 receptor activation is sufficient for ATP induction of SAPK activity, and suggests that the signaling pathway coupling P2X7 receptors to SAPK is not restricted to macrophages. Both p46 and p55 forms of SAPK in the HEK cells were similarly phosphorylated in response to P2X7 receptor activation (Figure 4B). This contrasts with the predominant accumulation of phosphorylated p46 isoforms in the ATP-pulsed BAC1 cells (Figure 3A). A preferential activation of p46 SAPK in mouse macrophages stimulated with TNFα has also been reported (57).

Dissociation of SAPK activation from increased caspase activity – Caspases are ubiquitously expressed cysteine proteases involved in the activation and execution of apoptosis (35). SAPK can be activated by the caspase-dependent cleavage of upstream
signaling molecules such as MEKK and Pak (36, 37). Since P2X7 receptor stimulation has been associated with activation of caspase-1 and caspase-3 in other inflammatory cell types (12, 18, 19), our findings suggested two possibilities: 1) ATP-dependent SAPK activity is the secondary consequence of caspase activation or 2) SAPK activity is upstream of caspase activation. We first verified that P2X7 receptor activation induced both caspase-3 and caspase-1 activity in BAC1 macrophages. The 30-minute ATP pulse induced time-dependent oligo-nucleosomal DNA laddering (Fig. 5A). Figure 5B shows that DNA laddering was preceded by the breakdown of a cytoskeletal protein, alpha-fodrin, which is a known substrate for caspase-3 during apoptosis (38, 39). Caspase-3 activity within cell lysates (measured by the hydrolysis of a fluorogenic substrate peptide) also increased as a function of time following the ATP pulse (Fig. 5C). The increase in caspase-3 activity at three hours closely correlated with the time course for alpha-fodrin breakdown, observed at 4 hours after the ATP pulse, supporting the notion that caspase-3 mediates alpha-fodrin proteolysis in these cells. The amount of caspase-3 activity in cell lysates also depended on the length of the ATP pulse (Fig. 5D) in a manner similar to that which characterized the accumulation of active SAPK within lysates (Fig. 2A, B). The caspase-3 inhibitor DEVD-cho attenuated DNA laddering in ATP-stimulated cells (Fig. 5C) and inhibited alpha-fodrin breakdown (Fig. 5D), supporting a role for caspase-3 in these events.

We also verified that activation of the P2X7 receptor could stimulate the rapid processing of proIL-1β to mature IL-1β in our system. In LPS-primed macrophages, an ATP stimulus triggered the release of processed IL-1 β, as measured by ELISA specific
mature IL-1β. Similar to previous reports, this process was mediated by the interleukin-converting enzyme (ICE)/caspase-1 because the caspase-1-like inhibitor YVAD-cmk strongly attenuated the ATP-stimulated release of immunoreactive IL-1β (Table I).

*P2X7 receptor*-dependent SAPK activity does not require caspase-1-like or caspase-3-like activity* Having established that ATP pulses can induce both caspase-3 and caspase-1 activity in these Bac1 macrophages, we next assessed whether ATP-induced SAPK activity was caspase-dependent. Figure 6 shows that at concentrations sufficient to inhibit their respective proteases, neither DEVD-cho nor YVAD-cmk had any effect on ATP-induced SAPK activity. These results indicate that the P2Z/P2X7 receptor couples to SAPK by a caspase-3 and caspase-1-independent mechanism.
Discussion

Despite strong evidence that the P2X7 receptor regulates macrophage apoptosis, IL-1β release and killing of intracellular mycobacteria, little is known regarding the signaling pathways that mediate these processes. The present study implicates SAPK as a novel P2X7 receptor effector and represents the first demonstration of stress-activated kinase signaling by this particular ATP-gated ion channel. Importantly, this activation of SAPK was triggered using brief pulses of extracellular ATP added to tissue culture medium containing physiologic levels of divalent cations, conditions designed to approximate the environment of a tissue macrophage.

Although millimolar levels of extracellular ATP were required to elicit SAPK activation, the pharmacologic and nucleotide selectivity data suggest that the P2X7 receptor alone specifically mediates this effect. While oxidized ATP has been extensively used as an effective P2X7 receptor antagonist, recent data suggests that this agent has multiple P2 receptor targets (40, 41). For this reason we also tested KN-62, an isoquinoline derivative and CamKII inhibitor first shown by Blanchard and colleagues to inhibit P2X7-dependent signaling (42). Gargett and Wiley demonstrated that this inhibition of P2X7 receptor function does not involve CAM-KII (43). We have documented a strong species-dependence to KN-62 sensitivity with the human P2X7 receptor exhibiting an IC50 of 30 nM, the mouse receptor being ~ten fold lower less sensitive, and the rat P2X7 receptor being virtually unaffected by this compound (33). Thus, inhibition by both oxidized ATP and KN-62 strongly suggests that the ATP-induced SAPK activity is mediated by the P2X7 receptor. The nucleotide selectivity
series also supports a role for this receptor as the sole mediator of SAPK activation. The requirement for millimolar ATP and the ability of submillimolar BzATP to mimic this effect of ATP typifies the agonist profile of the P2X7 receptor. Finally, the inability of a caspase-1 (interleukin-converting enzyme) inhibitor or a neutralizing anti-TNFα antibody to attenuate stimulation of SAPK by extracellular ATP argues against a role for secreted IL-1β or TNFα as indirect mediators of this ATP effect.

Although wildtype HEK293 cells natively express both P2Y1 and P2Y2 receptors (45), ATP pulsing did not trigger SAPK activation in these cells. Rather, heterologous expression of the P2X7 receptor in HEK293 cells appears necessary and sufficient to mediate the activation of SAPK by extracellular ATP in these fibroblast-like cells. This suggests that the biochemical pathway linking the channel/pore to SAPK is not restricted to macrophages. Whether P2X7 receptor expression is sufficient to reconstitute this pathway in other cellular backgrounds remains to be determined. Given recent data suggesting that the P2X7 receptor channel and the induced non-selective pore may be biochemically distinct entities (10), the SAPK-inducing signal could emanate from either the channel or the pore. It would be interesting to test whether SAPK can be activated by maitotoxin (MTX), a pore-inducing toxin that appears to couple to the same cytolytic pore as the P2X7 receptor (44).

P2X7 receptor ligation activates caspase-1 and caspase-3 in murine microglial cells and dendritic cells (18, 19), and our study confirms these findings in BAC1 macrophages. Caspase inhibitors abrogate induction of NFκB activity by P2X7 agonists so caspases have been proposed to mediate P2X7R-dependent NFκB induction (20). We hypothesized that the MEKK1 > MEK >
SAPK pathway might play a role in this signaling cascade because MEKK1 regulates activation of both SAPK and NFkB (24, 48). Moreover, caspase-3-dependent cleavage of MEKK1 into a 91 kDa fragment increases its kinase activity and could explain why P2X7-dependent NFκB induction is sensitive to caspase inhibition (27, 49). However, two lines of investigation strongly indicate that activation of caspase-1 and caspase-3-like proteases were not obligatory steps in the signaling pathways coupling P2X7 receptors to SAPK stimulation. First, SAPK activation preceded the accumulation of active caspase-3 by several hours. Second, inhibitors of either caspase-3 or caspase-1-like proteases had no effect on SAPK activation. These peptide inhibitors blocked caspase activity as reflected by their effects on alpha-fodrin breakdown and IL-1β release, indicating that sufficient concentrations of inhibitor were used to inactivate both proteases. One interpretation of these results is that SAPK lies upstream of caspase activation, and indeed SAPK has been shown to induce caspase activation (46). Alternatively, independent signaling pathways may link P2X7 receptors to the activation of SAPK and the various caspases. Induction of apoptosis itself is a form of cell stress that may trigger SAPK signaling without altering the execution of the cell death program (28, 47).

The mechanism by which P2X7 receptors trigger activation of the SAPK signaling cascade is unclear. Ca^{2+}-dependent activation of SAPK has been described (50). However, a simple rise in intracellular Ca^{2+} is an unlikely explanation for the ATP-mediated SAPK activation because UTP did not trigger SAPK despite its ability to
induce a sustained increase in Ca$^{2+}$ in BAC1 macrophages via activation of G protein-coupled P2Y2 receptors (34). Oxidative stressors are potent inducers of SAPK activity (53) and reactive oxygen intermediates accumulate in microglial cell stimulated by P2X7 receptor agonists (20). ATP-induced activation of SAPK in BAC1 macrophages was unaffected by the reducing agent n-acetyl cysteine (data not shown), making a role for reactive oxygen intermediates less likely. Depletion of bulk intracellular K$^+$ is implicated in SAPK and caspase activation, as well as apoptosis and necrosis (29, 30, 51, 54). Consistent with a possible role for K$^+$ efflux in mediating P2X7R activation of SAPK, inhibiting K$^+$ efflux in BAC1 macrophages (by replacing extracellular Na$^+$ with K$^+$) attenuated but did not eliminate the ATP-mediated SAPK activity (data not shown). Unequivocal interpretation of these experiments is difficult because this manipulation of the extracellular ionic conditions exerts striking effects on both the efficacy and potency of ATP as a P2X7 receptor agonist (52).

The identification of SAPK as a P2X7R effector opens new avenues for identifying the biochemical events that underlie processes involving the P2X7R such as MTB killing, apoptosis and IL-1β release. Future studies must address both the mechanism by which the P2X7R couples to SAPK and the physiologic consequences of activating this signaling pathway.
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References


Figure Legends

Figure 1. Treatment of murine macrophages with short pulses of extracellular ATP induces sustained activation of SAPK. Panel A, BAC1 macrophages were challenged with vehicle or with 5 mM ATP for 30 min at 37 ºC. The culture media was removed, the cells then washed once and fresh media added. Cells were then incubated at 37 ºC for the indicated times, lysed, and SAPK activity assessed as described in “Experimental Procedures.” Panel B, SAPK fold activation plotted as a function of time for the experiment shown in panel A. The solid bar from 0 to 30 min indicates the vehicle or ATP pulse.

Figure 2. Relationship between ATP pulse duration and induction of sustained SAPK activity. Panel A, BAC1 macrophages were challenged with vehicle or with 5 mM ATP for the indicated times, then the cells were washed once and fresh media added. After a 60 minute incubation at 37 ºC cells were lysed and SAPK activity. Panel B, SAPK fold activation plotted as a function of time for the experiment shown in panel A.

Figure 3. Activation of SAPK by extracellular ATP in macrophages is mediated by the P2X7 receptor. Panel A, BAC1 cells were incubated at 37º C in the absence (control) or presence of 5 mM ATP or anisomycin (200 nM) for 30 minutes. Cells were then washed once and fresh media was replaced lacking agonists (Control, ATP) or containing 200 nM anisomycin (Aniso). Cells were then incubated at 37º C for 60 min before lysis. Some cells were pre-incubated with the P2X7R-antagonist oxidized ATP (+oATP, 300 µM)
for three hours prior to stimulation. These groups were then challenged with ATP or anisomycin for 30 min in the continued presence of oATP. The oATP was not replaced after the washing step. Fold activation of SAPK was determined after quantitative PhosphorImager analysis of incorporated $^{32}$P into GST-Jun and normalized to the control lane. 30 µg of lysate from the same experiment was resolved by SDS-PAGE and analyzed by Western blot using either a polyclonal JNK antiserum ($\alpha$pan-SAPK) or a phospho-SAPK-specific antibody ($\alpha$P-SAPK).

**Panel B**, BAC1 cells were incubated at 37 ºC in the absence (control) or presence of 3 mM ATP or 200 nM anisomycin (Aniso) for 30 min. 5 µM KN-62 was added to some groups (+KN-62) along with the ATP or anisomycin. After 30 min the cells were washed once and fresh media replaced lacking agonists (Control, ATP) or containing 200 nM anisomycin (Aniso) in the continued presence of KN-62. After a 60 min incubation, cells were lysed and SAPK activity assessed and presented as in panel A. **Panel C**, the indicated nucleotides were added to BAC1 cultures for 30 min at 37 ºC. Cells were then washed once and fresh media lacking nucleotides was replaced. After the subsequent 60 min incubation, cells were lysed and SAPK activity was determined as described in panel A.

**Figure 4.** ATP-induced SAPK activity in wild-type vs. P2X7 receptor transfected HEK293 cells. **Panel A**, HEK-wt and HEK-P2X7 cells were plated in 6 well dishes coated with poly-L-lysine at 3 x $10^5$/ml one day before the experiment. Cells were challenged with 5 mM ATP for 20 min, then washed and incubated at 37 ºC for one hour and processed. Alternatively, cells were challenged with 10 µM anisomycin for 20
minutes then processed. SAPK activity was determined as described in Figure 1. Panel B, HEK-P2X7 cells in 6 well dishes were challenged with 5 mM ATP for 20 min, then washed and incubated in fresh medium for an additional 0, 30, 60, or 90 min prior to processing. Parallel wells were untreated or treated with 10 μM anisomycin for 60 min. 40 μg aliquots of lysate protein were resolved by SDS-PAGE and analyzed by Western blot using either a polyclonal JNK antiserum (αpan-SAPK) or a phospho-SAPK-specific antibody (α-phosphoSAPK).

Figure 5. Treatment of murine macrophages with short pulses of extracellular ATP induces sustained activation of caspase-3 and apoptotic induction. Panel A, BAC1 macrophages were pulsed with 5 mM ATP for 30 min, washed and incubated at 37°C for the indicated times. DNA cleavage products were isolated as described in materials and methods. Panel B, after a 30 minute, 5 mM ATP pulse, cells were incubated at 37°C for the indicated times. The ~240 kDa cytoskeletal protein alpha-fodrin was measured using Western blot analysis of cell lysates as described in materials and methods. The two major cleavage products at ~150 kDa and ~120 kDa result from caspase-3 dependent proteolysis (Martin 1995, Janicke 1998). Panel C, D, in vitro caspase-3-like protease activity was measured from cell lysates using a fluorogenic caspase-3 substrate peptide. Cytosolic lysates were made at the indicated times after a 5 mM ATP pulse of either 30 min (C) or various times (D). Panel E, the caspase-3-like inhibitor DEVD-cho (300 μM) was added to cultures 30 min prior to a 30 min, 5 mM ATP pulse. DEVD-cho was added back to cell cultures after washout, and DNA fragmentation assessed 6 hours after
ATP pulse. Panel F, alpha-fodrin breakdown was assessed 8 hours after a 5 mM ATP pulse (30 min) in the presence or absence of DEVD-cho (300 μM).

Figure 6. Caspase inhibitors do not repress ATP-induced SAPK activity. The caspase-3-like inhibitor DEVD-cho (300 mM) or the caspase-1-like inhibitor YVAD-cmk (200 μM) was added to BAC1 macrophages 30 min prior to challenge with ATP. 5 mM ATP was added, cells were incubated for 30 min at 37°C, then cells were washed with ATP-free media containing inhibitors and incubated for an additional 60 min. Cells were processed and SAPK activity determined as described in Figure 1.
**Table I.**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>[IL-1β] (ng/ml, avg ± range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>ATP</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>ATP + YVAD</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

**Table I. Inhibition of IL-1β release by YVAD-cmk.** BAC1 macrophages were treated with LPS (1 µg/ml) for 4 hours prior to challenge with 3 mM ATP for 60 minutes at 37°C. Where indicated, YVAD-cmk (200 µM) was added 30 minutes prior to ATP challenge. 5 µL of cell supernatants were then assayed for mature IL-1β by Elisa as described in Materials and Methods.
Fig. 1

A. 

Time (min): 0 30 60 90 150

Vehicle: -GST-Jun

ATP: -GST-Jun

B. 

Fold SAPK activity

Vehicle 
ATP

Time (min): 0 20 40 60 80 100 120 140 160

Fold SAPK activity vs. Time (min)
Fig. 2  

A.  

Pulse Duration (min)  

<table>
<thead>
<tr>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
</tr>
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<tbody>
<tr>
<td>vehicle</td>
<td>-GST-Jun</td>
<td></td>
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<table>
<thead>
<tr>
<th>0</th>
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<th>10</th>
<th>15</th>
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<th>30</th>
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<tbody>
<tr>
<td>ATP:</td>
<td>-GST-Jun</td>
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B.  

SAPK activity (fold stimulation)  

<table>
<thead>
<tr>
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<td>vehicle</td>
<td>ATP</td>
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**Fig. 3 A.**

<table>
<thead>
<tr>
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<th>+oATP</th>
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<tbody>
<tr>
<td>control</td>
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<td>Aniso</td>
</tr>
<tr>
<td>GST-Jun</td>
<td>fold: 1.0</td>
<td>5.7</td>
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**Fig. 3 B.**

<table>
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<tr>
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<tbody>
<tr>
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<td>Aniso</td>
</tr>
<tr>
<td>GST-Jun</td>
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**Fig. 3 C.**

<table>
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<tr>
<th>ATP (mM)</th>
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<th>1 mM BzATP</th>
<th>5 mM ADP</th>
<th>5 mM UTP</th>
<th>0.1 mM ADO</th>
<th>0.3 mM 2-meS-ATP</th>
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<tbody>
<tr>
<td>fold:</td>
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<td>1.7</td>
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- GST-Jun
- α P-SAPK
- α panSAPK
Fig. 4

A. HEK-wt

<table>
<thead>
<tr>
<th></th>
<th>HEK-wt</th>
<th>HEK-P2X7</th>
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</thead>
<tbody>
<tr>
<td>con</td>
<td>ATP</td>
<td>Aniso</td>
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</table>

- GST-Jun

B. Incubation post ATP pulse

<table>
<thead>
<tr>
<th></th>
<th>con</th>
<th>Aniso</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
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<tr>
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<td>pp55 SAPK</td>
<td>pp46 SAPK</td>
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</tr>
<tr>
<td>a-pan SAPK</td>
<td></td>
<td>p55 SAPK</td>
<td>p46 SAPK</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HEK-P2X7
Fig. 5

A. Time after pulse (hrs): markers 0 2 6 10

B. Time after pulse (hrs): 0 2 4 6 8 10

C. Caspase-3-like activity (arbitrary units)

D. Caspase-3-like activity (arbitrary units)

E. control ATP ATP + DEVD

F. control ATP ATP + DEVD
SAPK/JNK activation and apoptotic induction by the macrophage P2X7 nucleotide receptor

Benjamin D. Humphreys, Janet Rice, Sylvia B. Kertesy and George R. Dubyak

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